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Dynamic alterations of hepatocellular function by on-demand elasticity and roughness modulation†

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Temperature-responsive cell culture substrates reported here can be dynamically programmed to induce bulk softening and surface roughness changes in the presence of living cells. Alterations in hepatocellular function following temporally controlled substrate softening depend on the extent of stiff mechanical priming prior to user-induced material transition.

Subcellular substrate mechanics provide important cues towards regulating cellular function through mechanotransduction.[1,2] Although temporal variations in cellular microenvironmental compliance play an important role in regulating *in vivo* cell fate, current strategies for *in vitro* cell culture often fall short in their near-exclusive reliance on fully static materials. Recognizing this shortcoming, recent efforts have sought to create tunable cell culture platforms that exhibit dynamic biophysical properties.[3–5] Photo-, pH-, and molecular-responsive hydrogels enabling *in situ* modulation of substrate elasticity have been utilized to probe and direct changes in cellular morphology,[6] functional maturation,[7] and differentiation.[8,9] In addition to substrate mechanics, cells display a differential response based on physiochemical aspects of their microenvironment, including material surface wettability and swelling ratio.[10–12] To probe the effects of material stiffening and softening on cell function without the confounding aspects of differential swelling, non-swelling stimuli-responsive materials are needed.

We have previously reported the development of semicrystalline poly(ε-caprolactone) (PCL) films that exhibit dynamically tunable elasticity and topography while retaining similar surface wettability, and have used these for 2D cell culture.[13–17] Even though these studies dealt with substrates with supraphysiological properties, in terms of stiffness and roughness, the results indicated the possibility of a differential cellular response on these substrates depending on the cell type. To clarify this possibility, in this work, we further introduce a unique strategy to probe the temporal effects of substrate elasticity on cell function that exploits a temperature-responsive semicrystalline PCL elastomer. Taking advantage of the thermal response of a crosslinked semicrystalline elastomer, two different biomechanical states – one stiff and rough, and the other soft and smooth – can be obtained from the same material at physiologically relevant temperatures. We use these materials to examine hepatocellular function, which we find to be dependent on the timing of material alteration.

We first sought to design a crosslinked PCL film with a melting temperature (T_m) slightly above physiological temperatures, such that material crystallinity was preserved at 37 °C. This material was based on the thermally induced crosslinking of branched PCL macromonomers with end-group reactive acrylates. We successfully modulated the T_m of the PCL elastomer through nanoarchitectural specification, including extent of branching and the molecular weight of the PCL macromonomer [Table S1 and Fig. S1 (ESI†)]. The PCL elastomer was prepared by crosslinking two-branched (2b) and four-branched (4b) PCL macromonomers in the presence of a benzoylperoxide initiator.[13,18,19] The PCL elastomer with a mixing ratio of 70/30 wt% of 2b/4b-PCL macromonomers yielded a T_m of 38 °C and crystallization temperature (T_c) of 9 °C, as estimated by differential scanning calorimetry (DSC) [Fig. S2 (ESI†)].

To evaluate the temperature dependence of network mechanics, we performed tensile tests of the crosslinked PCL elastomer during both material heating and cooling [Fig. S3 (ESI†)].

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Sample temperature was maintained constant for 24 h to achieve thermal equilibrium of the materials prior to measurement, although we observed that heating above T_m, 41 and 43 °C for <1 h, was sufficient to induce a phase-transition of the substrate. As shown in Fig. 1(a), the elastic modulus (estimated from the initial slope of the linear portion of the stress–strain curve) decreased gradually with heating below 39 °C, but then dropped dramatically at 41 °C owing to melting of the crystalline phase. Interestingly, near-constant substrate elastic moduli were observed throughout material cooling after having been previously melted; a mechanical hysteresis between the heating and cooling process was observed. This thermal hysteretic response represents a ubiquitous phenomenon in a semicrystalline polymer system, as melting and crystallization generally take place at different temperatures. In other words, although crystallization-induced stiffening of materials should occur through cooling, this was not observed when samples were cooled from 43 to 30 °C, at least for 24 h. Importantly, the crosslinked PCL elastomers persisted as solids, even after heating above their T_m. Consequentially, two different mechanical environments were readily created from the exact same material compositions at 37 °C depending on past thermal treatments [Fig. 1(b)]. This represents a distinct advantage over many other systems whose network mechanics remain static and are defined by initial material composition.

In addition to mechanics, surface morphology and wettability also influence changes in cell behaviour.[10,20,21] Having previously characterized the temperature-dependent bulk properties, we next sought to evaluate the associated changes in surface morphology through atomic force microscopy (AFM). Fig. 2(a) shows topographic surface AFM images of crosslinked PCL elastomers at 37 °C during initial heating as well as during subsequent cooling. Irregular rough surfaces were observed at 37 °C during heating due to the presence of crystal domains. The values of mean roughness (R_a) at 37 °C were found to be 52.2 nm (20 × 20 μm^2). When the elastomers were heated at 43 °C, the surface became much smoother with R_a values decreasing to 10.3 nm (20 × 20 μm^2) [Fig. S4 (ESI†)]. Surface morphological smoothing is attributed to the phase transition of the material from a crystalline to an amorphous state. Surface smoothness persisted even as the material temperature was decreased; an R_a value of 16.1 nm (20 × 20 μm^2) was measured following heating above the T_m and subsequent cooling to 37 °C. These observations were consistent with the aforementioned results from DSC and tensile testing. Collectively, these experiments revealed that two different mechanical states could exist at physiological temperatures depending on whether or not the material had been heated above its T_m (43 °C).

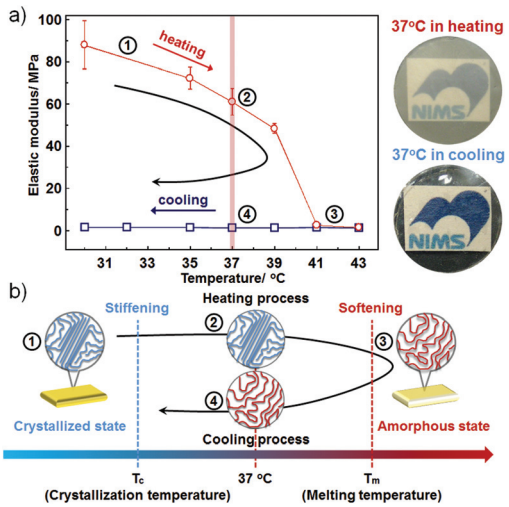

Fig. 1 (a) Temperature-dependent stiffness change of the crosslinked PCL elastomers with 70/30 wt% of 2b/4b-PCL macromonomers characterized by the tensile test. (b) Schematic illustration of the phase change of the PCL crystal and amorphous domains in response to temperature change of the heating and cooling process. The elastic moduli in each stage were (1) 87.9 ± 11.5 (at 30 °C), (2) 61.1 ± 6.2 (at 37 °C), (3) 1.4 ± 0.1 (at 43 °C), and (4) 1.4 ± 0.1 MPa (at 37 °C), respectively.

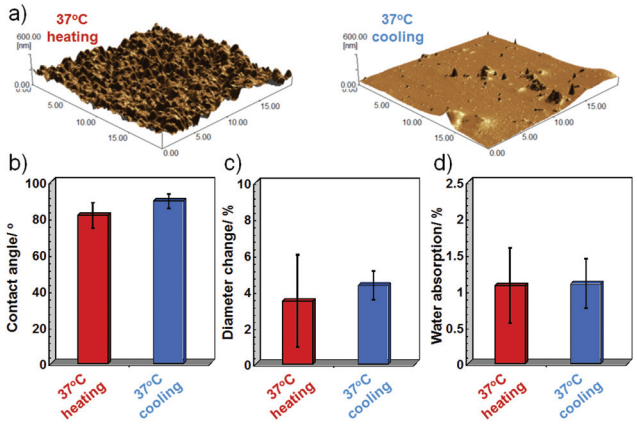

Fig. 2 (a) 3D AFM images of crosslinked PCL elastomers with 70/30 wt% of 2b/4b-PCL macromonomers at 37 °C in (left) the heating and (right) cooling process between 25 and 43 °C. The scan size is 20 × 20 μm and shown with a fixed height of 600 nm for each image. (b) Water contact angles at 37 °C (n > 6, data represent means ± SD) on crosslinked PCL elastomers corresponding to AFM images. Swelling behaviours, (c) diameter change and (d) water absorption ability of crosslinked PCL elastomers after immersion of PBS at 37 °C for 2 days (n > 3, data represent means ± SD).

To further characterize these temperature-responsive materials, we conducted contact angle measurements of crosslinked PCL elastomers at 37 °C [Fig. 2(b) and Fig. S5 (ESI†)]. The samples had similar contact angles of 81.6 ± 7.1° and 89.4 ± 4.1°, irrespective of thermal treatment prior to measurement. In addition to surface wettability, swelling behaviour of crosslinked PCL elastomers at 37 °C was determined [Fig. 2(c and d) and Fig. S6 (ESI†)]. Following immersion in phosphate buffer saline (PBS) for 2 days at 37 °C, we measured diameter changes in cylindrical discs of crosslinked PCL for each material state. The crystalline materials exhibited a 3.5 ± 2.6% increase in diameter, while amorphous materials displayed a 4.3 ± 0.8% change.

The water absorption (W_{abs}) of crosslinked PCL elastomers was calculated as:

$$W_{abs}(\%) = (W_{wet} - W_{dry})/W_{dry} \times 100$$

where W_{wet} is the weight of the wet specimen after removing surface water and W_{dry} is the weight of a completely dry sample (see the ESI†). Limited water uptake into crosslinked PCL elastomers was observed (~1% for both material states). Contrary to hydrogel cell culture platforms with stiffness changes accompanying both changes in material crosslinking and water content, these materials uniquely allow moduli to be tuned independently from swelling; this enables the effects of network mechanics and water content on cell behaviour to be decoupled, an important task that has proved challenging to achieve.[10–12] In addition, we observed that the protein adsorption onto stiff and soft crosslinked PCL was comparable [Fig. S7 (ESI†)], indicating that this non-swelling temperature-responsive material can be used to efficiently probe the cellular response against well-defined dynamic changes of material biomechanics, stiffness, and roughness.

The roles of surface elasticity and surface roughness in the cell culture substrate with exactly the same compositions on cell adhesion, proliferation, and functions on PCL elastomers were first investigated under static culture conditions at 37 °C. Human hepatocellular carcinoma cells, HepG2, were cultured on crosslinked PCL elastomers with different mechanics by pre-thermal treatment and tissue culture polystyrene (TCPS) dishes for up to 5 days at a static temperature of 37 °C [Fig. 3 and Fig. S8 (ESI†)]. HepG2 cells adhered and proliferated well on TCPS [Fig. S8 (ESI†)] as well as on stiff/rough and soft/smooth crosslinked PCL elastomers as shown in Fig. 3(a). No significant differences of adhesion and proliferation of HepG2 cells were observed between material conditions [Fig. S8 (ESI†)]; these results motivated us to further characterize additional biological functions. The synthesis and secretion of serum proteins represents one of the most important properties of hepatocytes; secretion of albumin remained roughly constant throughout the five-day culture, and was statistically indistinguishable between culture conditions within the expected range of secretion reported by others [Fig. 3(b)].[22,23] This suggests that both elasticity and surface roughness changes do not influence hepatocellular behaviour when cells are cultured under static conditions.

As the reported crosslinked PCL elastomers undergo dramatic changes in elasticity and surface roughness following mild heating (41 °C for 1 h), we anticipated that the crystalline state of these materials could be modulated in the presence of live cells. To investigate the hepatocellular response to dynamic material alterations, we quantified albumin secretion for HepG2 cells cultured on soft materials and materials whose stiff-to-soft mechanical transition was triggered at different points (1, 2, or 3 days after seeding, 5-day total culture) (Fig. 4(a)). Though cell morphology and proliferation were indistinguishable across all conditions, and no detachment was observed [Fig. S9 (ESI†)], significant changes to

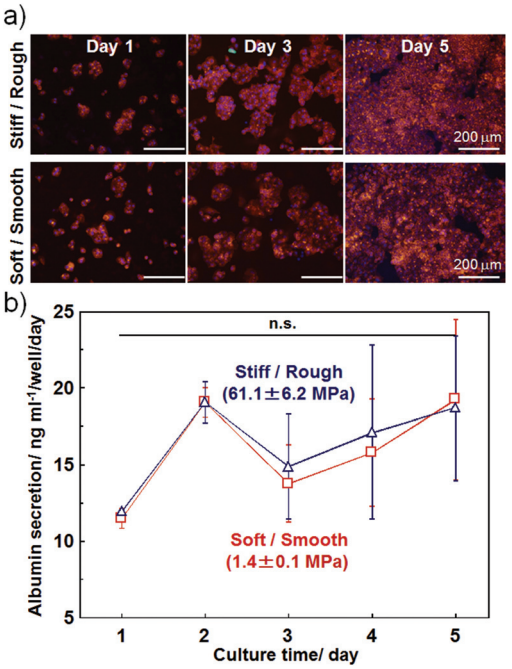

Fig. 3 (a) Immunofluorescence staining images of HepG2 cells on stiff and soft crosslinked PCL with 70/30 wt% of 2b/4b-PCL macromonomers at 37 °C. Immunofluorescence staining was performed against actin (red) and nucleus (blue) after cultivation for 1, 3 and 5 days. (b) Change of albumin secretion from HepG2 cells cultured on stiff (blue) and soft (red) crosslinked PCL elastomers at 37 °C. The amounts of secreted albumin were quantified by the ELISA assay after cultivation for 1, 3 and 5 days ($n > 3$, data represent means ± SD, n.s. not significant, t-test).

albumin secretion accompanied material softening. After heating-induced softening and smoothening of substrates, albumin secretion decreased at the time corresponding to material alteration (Fig. 4(b)). Albumin secretion for HepG2 cultured on soft substrates with or without heating (as indicated in red squares in Fig. 4(a) and 3(b), respectively), both of which present constant roughness and stiffness, showed no significant difference, indicating that a decrease in albumin secretion cannot be attributed to heating effects; instead, material dynamics associated with the stiff-to-soft transition are responsible for this functional change (as indicated by blue triangles in Fig. 4(a)). Additionally, mild heating to induce material alteration is expected to have minimal impact on the function of adhered cells.[24] Mechanical memory, which is the ability of cells to remember the past mechanical environment and depends on both culture time and substrate stiffness,[25,26] might be attributed to the different hepatocellular responses observed in this study. Therefore, dynamic regulation of hepatocellular function was achieved by temporal changes in material stiffness/roughness of crosslinked PCL elastomers in response to cytocompatible temperature changes. Though our current approach does not offer the ability to decouple the effects of stiffness and roughness on cell function, one possible strategy to do so moving forward could be to exploit a shape memory system where the stiff sub-

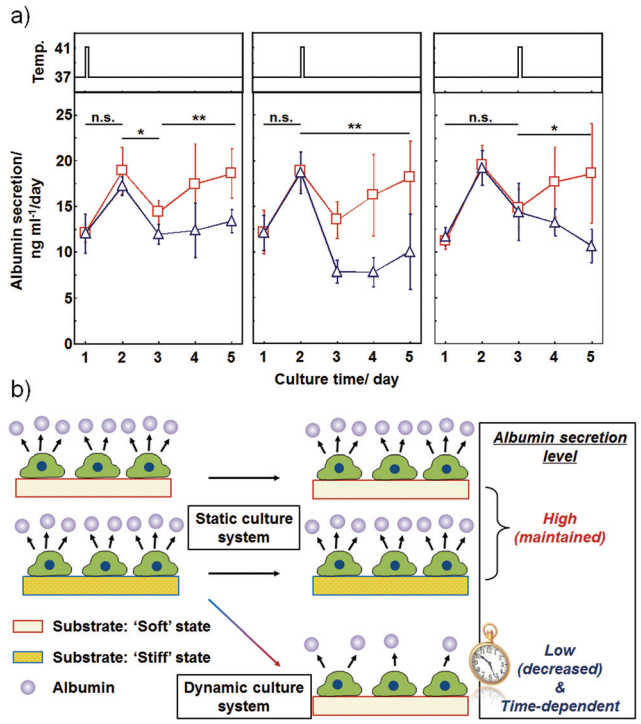

Fig. 4 (a) Time-dependent changes in albumin secretion from HepG2 cells cultured on stiff-to-soft (blue) and soft (red) crosslinked PCL elastomers with 70/30 wt% of 2b/4b-PCL macromonomers at 37 °C ($n > 3$, data represent means ± SD, n.s. not significant, $*p < 0.05$, $**p < 0.01$, *t*-test). Cells were cultured on the substrates at 37 °C for 1, 2 or 3 days. The HepG2-seeded materials were then subjected to heating at 41 °C for 1 h. (b) Schematic summary of hepatocellular albumin secretion ability in response to dynamic changes in substrate elasticity.

strate starts with a smooth surface (instead of the rough structures in the current studies) prior to transition to a soft smooth material.

In conclusion, a temperature-responsive crosslinked PCL elastomer was designed and applied as a novel dynamic cell culture substrate. Depending on the material crystallinity, which can be modulated with mild heating in the presence of live cells, two different material states can be achieved. HepG2 cells on the crosslinked PCL elastomer with modulatable stiffness and roughness altered their functions depending on the timing induced stiffness/roughness change. These results indicate that temporal modulation of substrate properties can induce dynamic changes in cell function.

Conflicts of interest

There are no conflicts to declare.

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